Studies on the Identification of Constituents in Ethanol Extract of Radix Glycyrrhizae and Their Anti-Primary Hepatoma Cell Susceptibility

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1. Introduction

Radix Glycyrrhizae is a legume, which is recorded in the Chinese Pharmacopoeia 2010 Edition Vol. I as the root and rhizome of Glycyrrhiza uralensis Fisch., G. inflata Bat., or G. glabra L. [1]. Radix Glycyrrhizae has a complex chemical composition, whose main constituents are flavonoids and saponins [2–4]. Despite extensive studies on chemical constituents of Radix Glycyrrhizae, pharmacological studies on monomeric compounds, especially the studies on anticancer activities and drug susceptibility, are still few [5]. To further improve the quality evaluation of Radix Glycyrrhizae and to better guide clinical medication, a systematic study of chemical constituents in Radix Glycyrrhizae is necessary, in order to obtain monomeric compounds and to study their antihepatoma activities.

Primary liver cancer is a common malignancy. Incidence of liver cancer is relatively high in many countries, and the majority of patients are not diagnosed until they are in the advanced stages. Liver cancer is relatively less sensitive to chemotherapy and is prone to drug resistance, which forms major reasons affecting chemotherapeutic effect on liver cancer. Among them, the multidrug resistance (MDR) of liver cancer not only limits the chemotherapeutic effect on liver cancer, but is also an important cause of recurrence and metastasis of liver cancer. Human hepatoma Bel-7402 cell line has become one of the commonly used in vitro biological models in the screening of novel anticancer drug candidates. Domestic scholars have used this model to screen out baicalin, cordycepin, melittin, ampelopsin, ligustrazine, and other natural active constituents with relatively high inhibitory activities on hepatoma Bel-7402 cells from natural medicines and explored their application values in adjuvant therapy, susceptibility, and other aspects of liver cancer [6, 7]. In this study, hepatoma tissues obtained from surgical resection were used to prepare and culture primary hepatoma...
cells, which were used to perform anticancer drug sensitivity test, with the aim of improving drug efficacy and reducing side effects.

2. Methods

2.1. Instruments. NMR spectra were determined using a Bruker AV-500 NMR Spectrometer. TLC silica gel GF-254 and column chromatography silica gel (200-mesh) were both manufactured by Xindy Chemical Plant in Yantai; RPTLC plates were products of Merck, Germany; Sephadex LH-20 was a product of Pharmacia; RP-18 reversed phase silica gel was a product of Great Door, Germany; MCI GEL CHP-20P was a product of Mitsubishi Chemical; and D101 macroporous resin is a product of Dashi Technology Development Co., Ltd. in Nanjing.

2.2. Drugs and Reagents. Radix Glycyrrhizae was purchased from Hebei Ecological Technology Co., Ltd., which was identified by Professor Deng Fei from the Beijing University of Chinese Medicine as the root and rhizome of Glycyrrhiza uralensis Fisch. All the reagents used were of analytical grade. Five cases of hepatoma tissues were provided by the Department of Pharmacy, General Hospital of Beijing Military Region of PLA, which had been pathologically resected hepatoma tissues were taken and soaked in sterile saline to make a 1% double-antibody, cut into 1mm³ pieces, then digested by addition of a 10-fold amount of 0.25% trypsin solution and 0.02% EDTA at 37°C for 35 min, and shaken every 5 min; digestion solutions were discarded, and the above H-NMR spectral data were consistent with the reported liquiritigenin data [8], so the compound was identified as liquiritigenin.

Compound 2: white powder, mp. 207–209°C. 1H-NMR (DMSO-δ6): δ: 10.66 (s, OH-7), 7.49 (2H, d, J = 8.4 Hz), 6.79 (1H, d, J = 8.6 Hz, H-5), 7.10 (2H, d, J = 7.2 Hz, H-3, 5‘), 6.50 (1H, d, J = 8.6 Hz, H-6), 6.34 (1H, s, H-8), 5.55 (IH, brd, J = 12.4 Hz, H-2), 2.76 (2H, m, H-3), 4.92 (1H, d, J = 6.2 Hz, H-1‘). The above H-NMR spectral data were consistent with the reported liquiritin data [9], so the compound was identified as liquiritin.

Compound 3: yellow powder. 1H-NMR (DMSO-δ6): δ: 13.66 (2’-OH), 8.19 (1H, d, J = 8.8 Hz), 7.79–7.70 (4H, α, β, 2, 6-H), 6.88 (2H, d, J = 8.4 Hz), 3.5-H), 6.38 (1H, dd, J = 8.8, 2.4 Hz), 3.6-H), 6.29 (1H, d, J = 2.4 Hz). The above data were basically consistent with the isoliquiritigenin reported in the literatures [10, 11], so the compound was identified as isoliquiritigenin.

Compound 4: white powder (chloroform). 1H-NMR (CDCl3): δ: 0.66, 0.74, 0.82, 0.88, 0.92, 1.66 (each 3H, 6xCH3), 4.08 (1H, H-3), 4.62, 4.75 (each 1H, C=CH), 4.3-NMR (CDCl3): δ: 38.6 (C-1), 27.6 (C-2), 76.9 (C-3), 38.7 (C-4), 55.3 (C-5), 19.0 (C-6), 34.3 (C-7), 40.7 (C-8), 51.0 (C-9), 36.9 (C-10), 20.8 (C-11), 26.4 (C-12), 37.2 (C-13), 43.4 (C-14), 31.5 (C-15), 32.1 (C-16), 56.3 (C-17), 49.5 (C-18), 46.8 (C-19), 150.4 (C-20), 29.7 (C-21), 38.2 (C-22), 28.0 (C-23), 15.1 (C-24), 15.9 (C-25), 16.1 (C-26), 14.9 (C-27), 179.9 (C-28), 109.9 (C-29), 19.4 (C-30). The above data were basically consistent with the reported literature [12], so compound 4 was identified as betulinic acid.

Compound 5: white powder (chloroform). 1H-NMR (CDCl3): δ: 0.76 (3H, s, H-23), 0.81 (3H, s, H-24), 0.90 (3H, s, H-25), 0.92 (3H, s, H-26), 0.93 (3H, s, H-29), 0.99 (3H, s, H-30), 1.13 (3H, s, H-27), 5.29 (IH, t, J = 2.4 Hz, H-12), 3.22 (IH, dd, J = 4.2, 11.4 Hz, H-3x), 2.84 (IH, dd, J = 13.8, 3.6 Hz, H-18); 13C-NMR (CDCl3): δ: 39.2 (C-1), 26.9 (C-2), 79.1 (C-3), 38.7 (C-4), 55.5 (C-5), 18.3 (C-6), 32.6 (C-7), 40.1 (C-8), 47.7 (C-9), 371 (C-10), 23.5 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.7 (C-15), 23.7 (C-16), 46.5 (C-17), 41.9 (C-18), 45.9 (C-19), 30.9 (C-20), 34.8 (C-21), 33.4 (C-22), 29.1 (C-23), 15.7 (C-24), 16.3 (C-25), 18.1 (C-26), 26.2 (C-27), 183.2 (C-28), 33.1 (C-29), 23.2 (C-30). The above data were consistent with the reported literature [13], and RF value and spot color on TLC were exactly identical with those of oleaneolic acid reference substance, so compound 5 was identified as oleanolic acid.

2.4. Anticancer Pharmacological Activity Test

2.4.1. Preparation of Primary Hepatoma Cells. Surgically resected hepatoma tissues were taken and soaked in sterile Hank’s solution; small amounts of nonnecrootic carcinoma tissues were selected, repeatedly washed in RPMI-1640 medium containing 1% double-antibody, cut into 1mm³ pieces, then digested by addition of a 10-fold amount of 0.25% trypsin solution and 0.02% EDTA at 37°C for 35 min, and shaken every 5 min; digestion solutions were discarded, and the
remaining was washed by addition of blank culture medium, then added with a small amount of culture solution, and repeatedly blown with a pipette into single cells, which were then passed through a 40-mesh sieve, and stained with 1% trypan blue, followed by counting of the number of viable cells.

2.4.2. MTT Colorimetry. \(2 \times 10^5\) hepatoma cell suspension was taken and seeded in 96-well plastic culture plates. Wells contained different drugs at 100 \(\mu\)L per well, with 90 \(\mu\)L added medium and 10 \(\mu\)L drug; the concentration of all drugs is 1 mg/mL. 10 replicate wells were set up for each drug; the plates were statically cultured in a CO\(_2\) incubator set at 37\(^\circ\)C for 24 h. After incubation, MTT reagent was added at 20 \(\mu\)L/well, and the cultivation was continued for an additional 6 h, then the supernatant was aspirated, and each well was added with dimethyl sulfoxide at 150 \(\mu\)L/well and shaken for 15 min, so that the MTT reduction product was completely dissolved. Absorbance (A value) of each well was measured at 570 nm using a microplate reader, and the proliferation inhibition rates against hepatoma cells by different drugs were calculated according to the following formula. Hepatoma cell proliferation inhibition rate = \(1 - \frac{A \text{ value of cells in treated wells}}{A \text{ value of cells in control wells}}\) × 100%.

2.4.3. Flow Cytometry. Changes in each cell cycle phase of primary hepatoma cells by different drugs were detected using flow cytometry. Cell culture and grouping methods were the same as “MTT colorimetry,” with the exception that 96-well plastic plates were replaced by 25 mL culture flasks; after cultivation, cells were collected and washed three times with PBS; supernatant was discarded and the remaining was fixed in 70% cold ethanol and stored within 4\(^\circ\)C; before being subjected to flow cytometry, the cells were passed through a 40-mesh sieve, concentration adjusted to \(1 \times 10^6\) cells/mL, and PI stained, followed by detection with flow cytometry; cell cycle results were analyzed using Modifi 2.0 software.

2.5. Statistical Processing. Data were analyzed using SPSS 11.0 statistical software, comparison of cell proliferation inhibition rate was performed by Student's \(t\)-test, and comparison of cell cycle between groups by \(F\) test. All data are expressed as mean ± standard deviation (\(\bar{x} \pm s\)).

3. Results

Positive control 5-FU is effective for inhibiting primary hepatoma cells. Comparing with 5-FU, liquiritigenin also had apparent proliferation inhibitory effects on primary hepatoma cells, while liquiritin and isoliquiritigenin had relatively low sensitivities to hepatoma cells, and betulinic acid and oleanolic acid had no clear effect on these cells. 5-FU and liquiritigenin could significantly inhibit hepatoma cell progression from G1 phase to S phase, where G0/G1 phase cells increased, S phase cells decreased, and G2/M phase cells relatively increased. See Tables 1 and 2.

4. Discussion

Ideal goal of adjuvant cancer chemotherapy is to pick out the most sensitive and most effective adjuvant chemotherapy regimens based on clinical manifestations, pathological type, and genetic characteristics of patients, thus ensuring maximum benefit for patients and achieving truly individualized treatment [14]. In this study, hepatoma tissues obtained from surgical resection were used to prepare single cell suspension and for primary cell culture, and drug sensitivity in cells was observed; MTT results showed that 5-FU and liquiritigenin had apparent inhibitory effects on primary hepatoma cells, with the inhibition rates of 42.9% and 35.7%, respectively, while the inhibition rates of drugs in other groups were not high. Difference in the sensitivity of various drugs was larger between the groups. Therefore, sensitivity of chemotherapeutic drugs should be tested before application to facilitate the individualized treatment for the same patient, in order to
reduce blind medication, thereby improving the quality of treatment and enhancing survival rate. MTT assay results can objectively reflect the activation state of tumor cells, because the MTT reagent can be reduced to blue formazan particles by dehydrogenases in the mitochondria of living cells of mammals, and the amount of formazan production is linearly correlated with the number of living cells and cell activation state; this experiment is widely used in the screening of anticancer drugs and the experimental studies of cytotoxicity. Flow cytometry results showed that different drugs all had effects on each cell cycle phase of primary hepatoma cells, but the sensitivities of 5-FU and liquiritigenin were higher. 5-FU converts into fluorouracil deoxynucleotide in the body and the sensitivities of 5-FU and liquiritigenin were higher. 5-FU group 10-

### Table 1: Primary hepatoma cell proliferation inhibition rates of various drugs (\(\bar{x} \pm s\), %).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration ((\mu)g/mL)</th>
<th>(A) value</th>
<th>Inhibition rate (%)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0</td>
<td>1.4 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-FU group</td>
<td>10</td>
<td>0.8 ± 0.2</td>
<td>42.9</td>
<td>&lt;0.01</td>
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<tr>
<td>Liquiritigenin group</td>
<td>100</td>
<td>0.9 ± 0.2</td>
<td>35.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liquiritin group</td>
<td>100</td>
<td>1.0 ± 0.0</td>
<td>28.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Isoliquiritigenin group</td>
<td>100</td>
<td>1.0 ± 0.2</td>
<td>28.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Betulinic acid group</td>
<td>100</td>
<td>1.3 ± 0.2</td>
<td>71</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Oleanolic acid group</td>
<td>100</td>
<td>1.3 ± 0.1</td>
<td>71</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Extract group</td>
<td>100</td>
<td>1.2 ± 0.3</td>
<td>14.3</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

### Table 2: Effects of various drugs on cell cycle phases of primary hepatoma cells (\(\bar{x} \pm s\), %).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration ((\mu)g/mL)</th>
<th>(G0/G1)</th>
<th>(P) value</th>
<th>(S)</th>
<th>(P) value</th>
<th>(G2/M)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0</td>
<td>26.7 ± 4.3</td>
<td>&lt;0.05</td>
<td>68.5 ± 3.2</td>
<td>&lt;0.05</td>
<td>5.8 ± 1.3</td>
<td>&lt;0.05</td>
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<tr>
<td>5-FU group</td>
<td>10</td>
<td>58.7 ± 3.6</td>
<td>&lt;0.01</td>
<td>26.7 ± 3.2</td>
<td>&lt;0.01</td>
<td>14.3 ± 3.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Liquiritigenin group</td>
<td>100</td>
<td>55.9 ± 4.1</td>
<td>&lt;0.01</td>
<td>28.7 ± 1.2</td>
<td>&lt;0.01</td>
<td>14.8 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Liquiritin group</td>
<td>100</td>
<td>39.8 ± 2.3</td>
<td>&lt;0.05</td>
<td>49.2 ± 1.1</td>
<td>&lt;0.05</td>
<td>11.2 ± 0.6</td>
<td>&lt;0.05</td>
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<tr>
<td>Isoliquiritigenin group</td>
<td>100</td>
<td>38.5 ± 3.1</td>
<td>&lt;0.05</td>
<td>49.3 ± 1.5</td>
<td>&lt;0.05</td>
<td>12.3 ± 0.4</td>
<td>&lt;0.05</td>
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<tr>
<td>Betulinic acid group</td>
<td>100</td>
<td>30.7 ± 3.2</td>
<td>—</td>
<td>63.5 ± 2.0</td>
<td>—</td>
<td>6.5 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>Oleanolic acid group</td>
<td>100</td>
<td>28.5 ± 3.1</td>
<td>—</td>
<td>64.6 ± 1.6</td>
<td>—</td>
<td>6.8 ± 0.2</td>
<td>—</td>
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### References


